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THE CELL RECEPTOR IN HYPERSENSITIVITY

ROBERT DAVID ARBEIT

1972

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The Cell Receptor in Delayed Hypersensitivity

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B.A., Williams College, 1968

Submitted to the faculty of the School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Medicine.

Yale University School of Medicine

Department of Medicine

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Introduction

Survival is a function of the ability of an organism to fill its ecological niche efficiently and to defend itself against aggression. The evolution from unicellular through oligocellular to multicellular forms with highly differentiated organ systems involves many obvious advantages. But there are also disadvantages. Multicellular organisms may be hosts for unicellular and subcellular pathogens; moreover, the processes of rapid proliferation and extensive differentiation create increased opportunities for somatic mutation and the development of uncontrolled neoplastic cell lines. It is teleologically reasonable, albeit unproven, that defenses should develop to meet these challenges.

In 1882 Metchnikoff proposed that phagocytic cells provided significant protection against microbial infections. He showed that phagocytosis is an important defense in a wide range of species -- from amoeba and colonial protozoa where phagocytic and digestive functions are combined, through higher forms in which wandering cells can be mobilized for use in any part of the organism (Humphrey & White, 1970). Metchnikoff's theory was strongly attacked by those who stressed the contribution of specific humoral defense factors. For many reasons, including methodological difficulties, specific cell-mediated defenses were only slowly appreciated. In

1891 Koch described the delayed time course of a skin reaction elicited by an intradermal tuberculin test in a sensitized host. In following years the perivascular mononuclear cell infiltrate characteristic of these lesions was noted (reviewed by Uhr, 1966). Landsteiner in 1942 showed that while serum alone could not transfer the ability to produce a delayed skin test, peritoneal exudate cell could. Further work by Chase and others established that lymphoid cells were the necessary and effective component of such an "adoptive transfer" (Bloom & Chase, 1967). Landsteiner also developed "artificial antigens" -- well-defined small molecules (haptens) attached to proteins (carriers). Using these he demonstrated that humoral antibody of marked hapten specificity could be elicited (Landsteiner, 1945). In contrast, delayed hypersensitivity was shown to display carrier specificity (Benacerraf & Gell, 1959).

These four characteristics -- the delayed time course and the histology of the in vivo reaction, transferability by living lymphoid cells but not by serum, and carrier specificity -- have remained the basic criteria for defining delayed hypersensitivity. Classical tubercular hypersensitivity and contact allergy are now considered two examples of cell-mediated immunological reactions; others include immunity to intracellular parasites, homograft rejection and

tumor rejection. The operative cells belong to a sub-population of lymphocytes dependent on the presence of the thymus and referred to as T-lymphocytes. The other functionally defined population of cells is called B-lymphocytes. These differentiate, proliferate and mature into plasma cells which synthesize humoral antibody.

Specifically sensitized T cells are directly cytotoxic for target cells and when exposed to antigen, they release soluble factors which effect vascular permeability, macrophage activity and other lymphocytes (Roitt, 1969). These responses seem to be the mechanism for the observed in vivo phenomena and have become the basis for a host of in vitro assays. In addition, T cells may cooperate with precursors of antibody-producing cells during the immune response to certain antigens ("helper" cell function).

In clinical and experimental situations where the thymus is congenitally absent, there is a defect in the ability of the organism to mount an immune response. Homografts are not rejected, contact sensitization cannot be achieved and humoral antibody reponse to a variety of protein antigens is diminished. Patients with such defects are highly vulnerable to opportunistic viral and fungal infections and the natural history of the

disease is brief and fatal. Clinically and immunologically, depletion of cell-mediated immunity (CMI) is a much graver defect than absence of humoral antibody (Fudenberg et al, 1971). The class of lymphocytes associated with CMI, the T cells, seem intimately related to the capacity for organizing an immune response, particularly to proteins, which are apparently the most relevant naturally-occurring antigens.

The clonal selection theory of immunology postulates that immunocompetent lymphocytes are pre-committed to respond to specific antigens. It seems likely that the initiation of cellular activity for an immune response is mediated by the interaction of antigen with specific cellular receptors. However, the receptors for T-lymphocytes are only poorly characterized in structural or functional terms.

One approach to the study of T-cell receptors is through affinity labelling, a technique which has been very useful in the study of antibody combining sites (Singer, 1967). The method depends upon the ability of a bifunctional reagent to (1) combine specifically with the particular antibody, and (2) react readily with amino acid residues to form covalent bonds. Hill (1971) reported that a hapten-derived affinity label could interfere with T-cell function and could permit

the isolation of a putative receptor. The first part of this study is an attempt to reproduce that system.

Whereas humoral antibody can be hapten specific, cellular immunity consistently shows "carrier specificity". It would be useful to have a system which would facilitate more careful investigation of this requirement of T-cell function. The relevant "carrier" determinants of classical hapten-protein antigens cannot be precisely identified. A defined sequence polypeptide antigen, however, could be used to study the specificity of the cell receptor and the requirements for immunogenicity. Further, such an antigen could serve as the basis of a "carrier-derived" affinity label. Thus, the second part of this study involved the synthesis of a defined sequence polypeptide antigen.

Materials, Methods and Results

I. Efforts to Synthesize a Hapten-Derived Affinity Label (after Hill, 1971)

Materials.

ϵ-2,4-dinitrophenyl-L-lysine (DNP-lysine) was a gift of Dr. William Konigsberg (prepared by the method of Porter, 1950). Na_2S was stored at 4° C. The "reducing solution" was: $Na_2S \cdot 9H_2O$ (4.8g, 20mmol) plus $NaHCO_3$ (1.68g, 20mmol) in H_2O (10ml) at 40° C (after Hodgson & Ward, 1945).

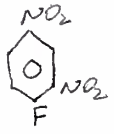
Reduction of DNP-lysine (after Hodgson & Ward, 1945).

ϵ-DNP-L-lysine HCl (348.5mg, 1mmol) (I in Fig. 1) was dissolved in methanol (10ml), neutralized by dropwise addition of 10N NaOH, and warmed to 55° C. The reducing solution was added dropwise until in excess as detected by ferrous sulfate paper. The solution was then cooled to 0° and the precipitate filtered and recrystallized from ethanol. The clean tan crystals were shown to have one nitro group reduced to an amino group (see below). Which group was reduced is unclear, but could probably be established by NMR studies. The product is called ϵ-2,4-(nitro, amino) phenyl-L-lysine (NAP-lysine) (II).

Figure 1

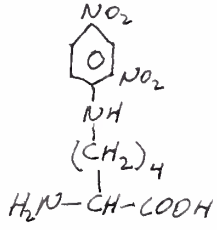
Dinitrophenyl Derivatives

I DNFB



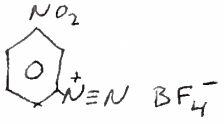
1-fluoro-2,4-dinitrobenzene

II ϵ-DNP-lysine



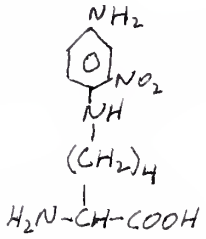
ϵ-2,4-dinitrophenyl-lysine

III m-NBDF



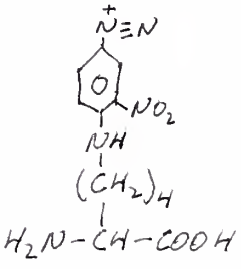
m-nitrobenzenediazonium fluoroborate

IV ϵ-NAP-lysine



ϵ-2-nitro-4-aminophenyl-lysine

V ϵ-NDP-lysine



ϵ-2-nitro-4-diazophenyl-lysine

Evidence for Formation of NAP-lysine.

(1) Electrophoresis. (Conditions given and results diagrammed in Fig. 2). The results indicate the presence of an additional positively charged group on NAP-lysine.

(2) Formation of a colored azo dye. NAP-lysine (approx 5mg) in H_2O (1ml) was chilled in an ice-bath and concentrated H_2SO_4 (3-4 drops) added. Chilled 1% $NaNO_3$ solution was then added dropwise until in excess as indicated by starch-KI paper. Dropwise addition of this solution to 2-napthol in 10% NaOH yielded formation of a red-brown precipitate. This is presumed to be an azo dye, thus implying the presence of a diazotizable amino group on the starting material. Negative controls included (1) starting with DNP-lysine and (2) omitting $NaNO_3$ solution.

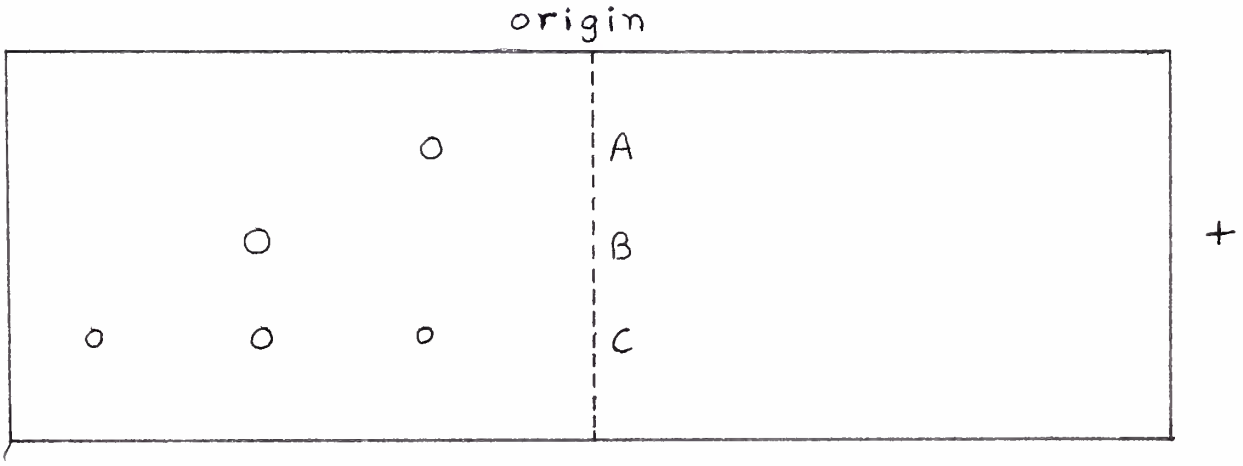
(3) Mass spectroscopy. The molecular weight of the product was not successfully established due to technical difficulties.

Binding of NAP-lysine to anti-DNP antibody.

Anti-DNP antibody was isolated from rabbit immune sera (approx 1mg Ab/ml) by the method of Eisen (1964a). Two stock solutions of antibody in 0.1M Tris (pH7.6) were prepared: (a) 0.480mg/ml) for use in equilibrium dialysis and (b) 0.047mg/ml for use in fluorescence quenching.

Figure 2

Electrophoresis of NAP-lysine



Conditions. pH 2.3; buffer, formic acid : glacial acetic acid : water :: 3.3ml of 98% soln : 29ml : 2000ml; voltage, constant at 250v; amperage, approx. 7mamp; time, 90min.

Notation: A = DNP-lysine (starting material)

B = NAP-lysine (recrystallized)

C = crude reaction mixture

Results and Interpretation.

The product, NAP-lysine (B), contains more positive charge than the starting material, DNP-lysine (A). If the difference in movement is interpreted as linearly proportional to charge, then B has twice as many positive charges. That is, B has two positive charges to A's one. We presume that the additional positive charge results

Figure 2 (continued)

from the reduction of a nitro group to an amino group.

The crude reaction mixture (C) contains not only (A) and (B) but also an additional side product which moves further toward the cathode. This has an even greater positive charge and presumably represents the doubly reduced diaminophenyl compound.

Equilibrium dialysis (Eisen, 1964b) was performed with micro-dialysis cells (Gateway Immunoserum Co., Illinois). ^{3}H,ε-2,4-DNP-L-lysine (New England Nuclear Co., Mass.) of specific radioactivity 2.57 x 10^{13}cpm/mole was a gift of Dr. R.W. Rosenstein. With this a stock solution of 1 x 10^{-5}M ^{3}H-DNP-lysine in Tris buffer was prepared. The dialysis cells used 50µl on each side of the membrane. One side received 50µl of antibody stock solution. When evaluating the antibody, the other side received 5,10,15,25 or 50µl of hapten solution plus (50-x)µl of buffer. When evaluating inhibition by ε-NAP-L-lysine, this side received 10µl (or 15µl) of DNP-lysine solution plus 40µl (or 35µl) of 10^{-5}, 10^{-4}, 10^{-3} or 10^{-2}M NAP-lysine solution. The cells were equilibrated overnight and then 25µl of each side was counted on a scintillation counter.

The results of these three determinants showed that the antibody preparation had a K_o of about 3.0 x 10^{6} $(M/L)^{-1}$ and an r of about 1.1. NAP-lysine, even at over 1,000-fold excess, did not interfere with DNP-lysine binding.

Fluorescence quenching was done by the method of Eisen (1964c). The stock solution of DNP-lysine used was 1 x 10^{-4}M. The 3ml cell was filled with an antibody solution and the hapten solution added in 10µl increments.

The results of two determinations give a K_o and r for the antibody preparation in the same range as that noted above.

The following experiments were performed using an NAP-lysine solution of 1 x 10^{-2}M. (1) NAP-lysine, in 10µl increments up to 100µl total, produced only a slow linear non-specific quenching of fluorescence, not the sharp initial drop indicative of specific binding. When DNP-lysine was then added, a classic quenching curve was obtained. The K_o and r calculated from this curve were remarkably consistent with previous results, despite the presence of 100-fold excess of NAP-lysine. (2) NAP-lysine and DNP-lysine aliquots were added alternately to the antibody solution. Quenching was observed only when DNP-lysine was added. There was no increase or decrease in fluorescence when NAP-lysine was added. Thus DNP-lysine caused normal fluorescence quenching despite the presence of 100-fold excess of NAP-lysine.

In summary, there was no evidence that ϵ-NAP-lysine could bind to anti-DNP antibody. Affinity labelling of antibody or immune cells with the diazo-derivative was not attempted.

II. Preparation of a Synthetic Polypeptide Antigen

Materials.

ϵ-DNP-L-lysine HCl was obtained as above. NCA-L-tyrosine and NCA-D,L-alanine (Pilot Chemical Co., Watertown, Mass.) were stored in a vacuum desiccator at -20° C. Absolute ethyl alcohol , reagent grade, and dry HCl gas (Matheson Co Inc., New Jersey) were used in the synthesis of ϵ-DNP-L-lysine ethyl ester HCl. Gel chromatography was performed with Sephadex G-25 and G-50 (Pharmacia Fine Chemicals, Sweden) and Bio-Gel P-2 (Bio-Rad Laboratories, Calf.).

Rationale and Principles of the Method of Synthesis.

For reasons that will be elaborated in the discussion, the antigen desired for this study was:

$$(\text{D-ala-L-ala-D-ala-L-tyr-D-ala-L-ala-D-ala-L-tyr-D-ala-L-ala-D-ala-}\epsilon\text{-DNP-L-lys})_{10}$$

The procedure of choice for synthesizing the monomeric subunit is the Merrifield solid-state method (Merrifield, 1969). However, this method is very time-consuming.

An alternative procedure involves the use of N-carboxy-α-amino acid anhydrides (NCAs) (Katchalski & Sela, 1958). These amino acid derivatives have been employed extensively to make synthetic polypeptides for

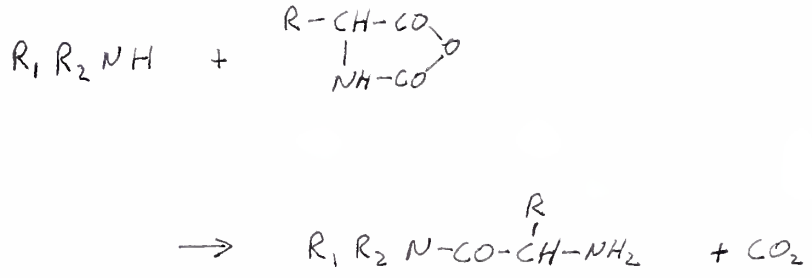
immunological studies (Sela, 1966; Sela, 1969). The polymerization of NCAs in inert solvents (e.g. dioxane) may be initiated by primary amines (e.g. amino acid esters or peptides). Polymerization under these conditions may be described by an initiation reaction (Fig. 3a) and a propagation reaction (Fig. 3b).

The occurrence of the initiation reaction formulated has been demonstrated by the disappearance of the initiator from the reaction mixture and the simultaneous appearance of the corresponding amino acid amide during the early stages of polymerization. The latter contains a free α-amino group and may, therefore, continue to react with available NCA. Dimers, trimers and higher peptides are formed by successive propagation steps and grow continuously until the reaction ceases because of either exhaustion of the NCAs or the disappearance of the terminal amino groups by a termination reaction (i.e. side reactions which diminish the number of free α-amino groups in the system). The occurrence of a propagation reaction of the type given above is supported by the observation that the polymerization of different NCAs can be initiated by peptides and by preformed polyamino acids containing free α-amino groups. Furthermore, no change in the concentration of free amino groups occurs in the polymerization devoid of a termination reaction.

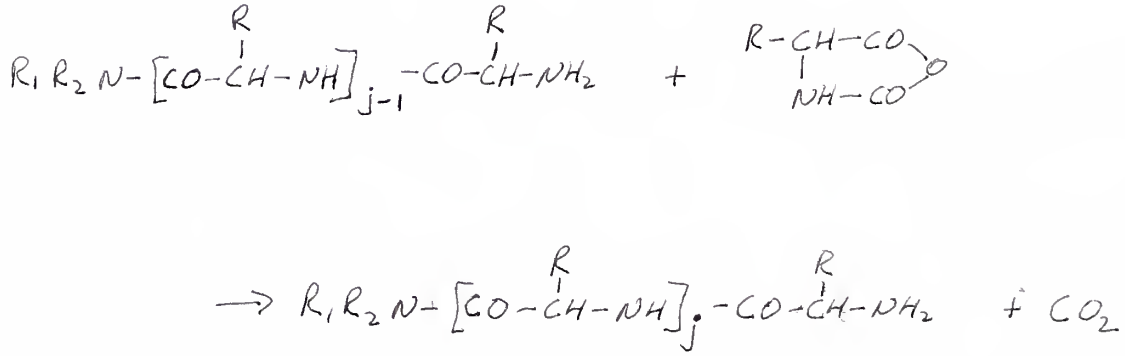
Figure 3

Description of NCA Reactions

(a) Initiation.



(b) Propagation.



In the polymerization scheme proposed above, growing chains react only with free NCAs and not with each other. Therefore in the absence of a termination reaction, which may usually be neglected at room temperature, a narrow Poissonian molecular weight distribution may be expected. Thus, the peptides formed would be predicted to have a length determined by the starting ratio of NCAs to initiator, and a composition reflecting the original mixture of NCAs. The sequence of amino acid residues is uncontrolled and should be, to a considerable degree, random (Katchalski & Sela, 1958). Thus, using the NCA procedure, a model compound could be synthesized more easily than the ideal one, though it would be less well defined.

The task was to synthesize a peptide approximating

$$(D,L\text{-ala}_{10},L\text{-tyr}_{2})\text{-}\epsilon\text{-DNP-L-lys}$$

by using ϵ-DNP-L-lysine as an initiator of an appropriate mixture of D,L-ala-NCA and L-tyr-NCA. The most satisfactory procedure was to synthesize the ethyl ester of ϵ-DNP-L-lysine HCl, neutralize it, and use the ethyl ester free base as the initiator. An example of this procedure is detailed below; for other systems and further details, see Appendix.

Synthesis of the Initiator.

ϵ-DNP-L-lysine HCl (200mg, 0.57mmol) was suspended in ethanol (4ml) and a stream of HCl gas passed through rapidly, without external cooling. After the amino acid dissolved, the hot reaction mixture was cooled in an ice bath, and the introduction of gas continued to saturation (approx. 2 hours). The reaction mixture was then sealed and let stand in a desiccator overnight at room temperature. By the next day the reaction had gone virtually 100% as determined by silica gel thin layer chromatography (Buffer -- butanol: acetic acid: water: pyridine::15:3:12:10). Dry nitrogen gas was passed through to drive off much of the excess HCl and then the solution was dried in vacuuo over sodium hydroxide. The yellow residue was recrystallized from ethanol and ether and dried in vacuuo again. The yield of ϵ-DNP-L-lysine ethyl ester HCl was approximately 90% (193mg, 0.513mmol) (Greenstein & Winitz, 1961).

This product (105mg, 0.30mmol) was dissolved in 50% ethanol (4ml), neutralized with Na_2CO_3 (0.15mmol) and dried in vacuuo, leaving a residue of ϵ-DNP-L-lysine ethyl ester free base plus an equimolar amount of NaCl. The residue was not soluble in dioxane or THF, either separately or mixed. It was largely soluble in benzene-dioxane (3.0ml plus 0.5ml), although a noticeable

amount of yellow, almost syrup-like flocculate was present. This mixture was used to initiate the polymerization reaction.

Synthesis and Isolation of a Peptide (SAM-4).

NCA-D,L-alanine (484mg, 4.2mmol) and NCA-L-tyrosine (186mg, 0.9mmol) were dissolved in dioxane (3.0 - 4.0ml). The NCAs dissolve completely in dioxane; a cloudy solution indicates some decomposition to free amino acid. The NCA solution and initiator solution were mixed and stirred overnight at room temperature. The next day an equal volume (7ml) of 1N NaOH was added. The solution now became clear and was stirred another two hours. These conditions had been shown, using silica gel thin layer chromatography, to completely de-estrify ϵ-DNP-L-lysine ethyl ester. After two hours the solution was neutralized (6N HCl) and lypholyzed.

Aliquots (100mg) of the lypholyzed powder were dissolved in sodium phosphate buffer (0.01M, pH7.4; 4ml). Insoluble material was separated by centrifugation and the supernatant chromatographed on G-25 Sephadex (Fig. 4).

The OD_{360} and OD_{278} of the fractions were measured. The former is the E_{max} for ϵ-DNP-L-lysine (ϵ=17,400); the latter is influenced strongly by

Figure 4

SAM-4: Gel Chromatography Fractionation

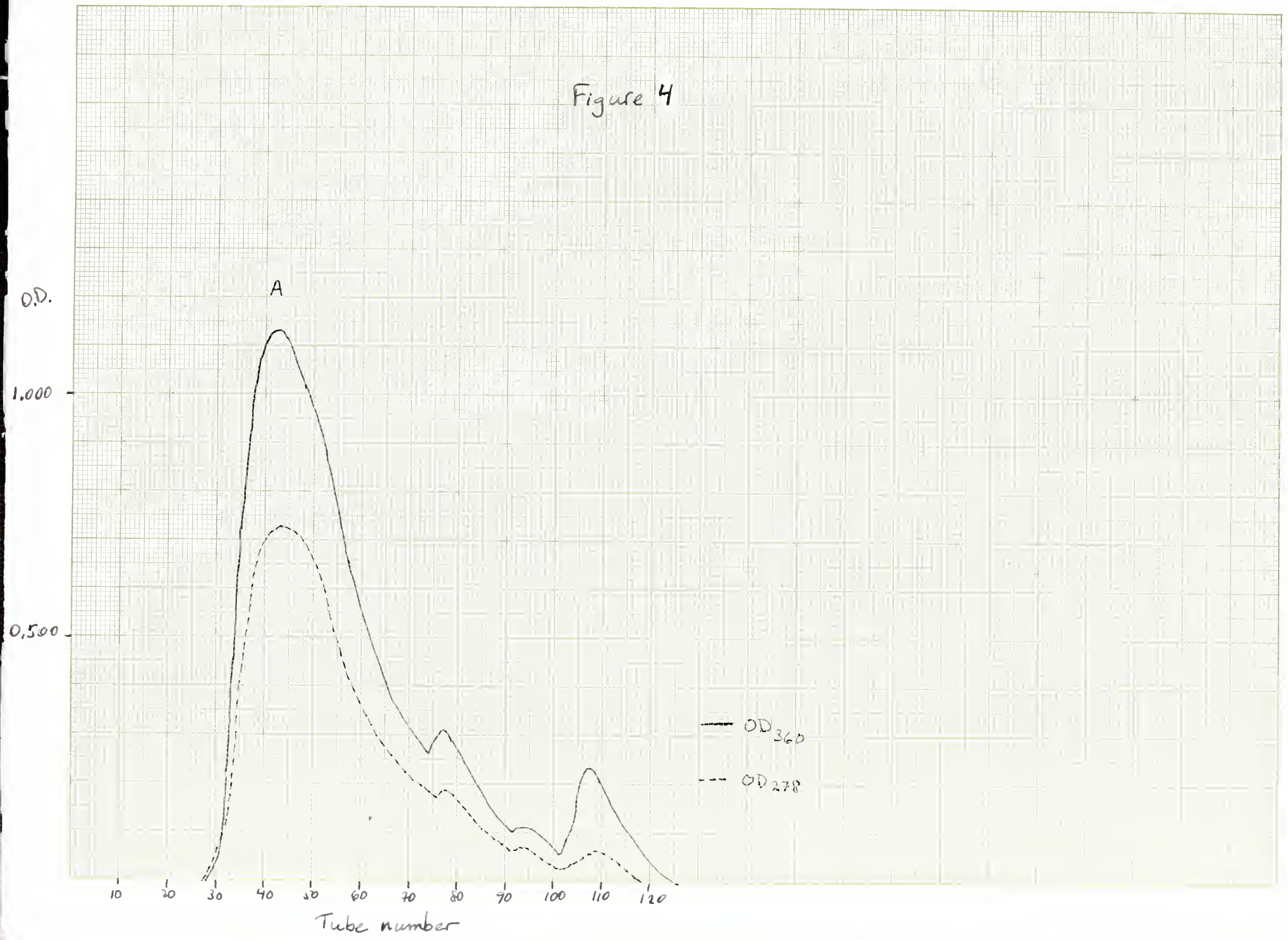
OD_{360} and OD_{278} vs Tube number

Conditions: G-25 Sephadex column (2.8cm x 55cm) eluted with PBS (Phosphate Buffered Saline: 0.01M Phosphate, pH7.4, 0.155M NaCl). Each tube represents 4.9min collection (approximately 5ml).

Results: By spectroscopic analysis.

Peak (Tubes)	Average Composition (D,L-ala:L-tyr: -DNP-L-lys)
SAM-4A (32-62)	14 : 3 : 1

Figure 4


O.D.
A
1.000
0.500
OD_{360}
OD_{278}
10
20
30
40
50
60
70
80
90
100
110
120
Tube number

the DNP group ($\epsilon \cong 3{,}000$), but is also the isosbestic point for tyrosine ($\epsilon = 1{,}350$) (Greenstein & Winitz, 1961). A graph of both OD's against fraction number provides an impression of the variation in the relative amount of tyrosine per DNP group throughout the gel separation. That is, if the two curves are parallel, a peak is more likely to represent a homogeneous population of peptides of relatively constant tyrosine:DNP content (e.g. SAM-4, Fig. 4); if the two curves are not parallel, there are likely to be several overlapping populations of peptides of different composition (e.g. SAM-3, see Appendix). Fractions were pooled, lypholyzed, desalted on a Bio-Gel P-2 column, and lypholyzed again. The product was then either polymerized or used directly for immunization.

A more precise determination of the average amino acid composition of an isolated peak was made by either amino acid analysis or spectrophotometry. For the former, a small aliquot was dissolved in 6N (constant boiling) HCl, sealed under vacuum in an ampule, heated to 110° C for 16 hours, dried in vacuuo and dissolved in analyzer buffer (0.01N HCl). The ϵ-DNP-L-lysine content of an aliquot was determined from the OD_{360} and the alanine and tyrosine content from the amino acid analyzer. For the spectrophotometric method, an aliquot

of peptide was diluted in 0.1N NaOH. The ϵ-DNP-L-lysine content was determined from the OD_{360} (λ_{max}, ϵ=17,400), and the tyrosine content from $OD_{293.5}$ (λ_{max}, ϵ=2,330) after correction for the DNP contribution ($OD_{293.5}/OD_{360}$ = 0.170). The alanine content was estimated by assuming an alanine:tyrosine ratio equal to that of the initial reaction mixture.

Synthesis of the Polymer.

The pentachlorophenyl active ester polymerization method was used (Kovacs et al, 1966; Richards et al, 1967). The peptide monomer (SAM-4A, 15μmol) was dissolved in a minimum volume of dichloroethanol (0.3ml) and a 1.1 molar excess of pentachlorophenol added. The mixture was cooled to 0° C and a 1.1 molar excess of triethylamine and N,N'-dicyclohexylcarbodiimide added. It was then shaken intermittently at 0° C for 12 hours, and dried in vacuuo at 36° C. Unfortunately, chromatography on Sephadex G-50 and G-25 gave no evidence of polymerization. There was insufficient time to perform quantitative dansylation and Edman degradation. This procedure would determine the number of N-terminal amines available per DNP group and thus the degree of polymerization. The inability to polymerize the peptide is probably a result of the low concentration of the reaction solution.

Both reports cited above required 1M concentrations of monomer to achieve polymerization. Because of low yields, only 0.05M monomer was present in our reaction mixture.

Immunization and Challenge.

Four to six male and female Hartley strain guinea pigs, weighing 350 - 400g, were used for each peptide tested. Suitable dilutions of peptides (500mg/ml) were emulsified with an equal volume of complete Freund's adjuvant (Difco Labs, Inc., Michigan). Each animal received 100mg of peptide distributed in the four foot pads in a volume of 0.1ml per foot pad. Eight days later, the guinea pigs were skin tested intradermally in one flank with 10mg of peptide in 0.1ml of isotonic saline and in the other flank with 10mg of PPD. On day 10, they were challenged with 50mg of peptide. Non-immunized control pigs were similarly challenged with peptide. Reactions were read at 4hr and 24hr following challenge. Two perpendicular diameters of the reaction site were measured and averaged.

No immediate reactions were seen. All immunized guinea pigs reacted to PPD with more than 10 x 10mm of erythema and induration, most with more than 14 x 14. No immunized pigs had more than 4 x 4mm of erythema and

induration to any peptide tested. A similar amount of reaction was seen in the control pigs. Biopsies of the challenge site of two immunized pigs revealed only non-specific polymorphonuclear leukocyte infiltrate with no mononuclear cells or perivascular cuffing. A list of the peptides used for immunization, their code and their composition is given in Table I.

Table I.

Peptides Tested for Immunogenicity

Code Name	Average Composition (D,L-ala:L-tyr: -DNP-L-lysine)	Approximate Molecular Weight
SAM-2E	10.8 : 2.3 : 1	1200
SAM-3A	18.2 : 4.0 : 1	2100
SAM-4A	14.0 : 3.0 : 1	1600
SAM-4A-P*	$(14.0 : 3.0 : 1)_{?n}$	(1600)x(?n)

*The products of the polymerization procedure were tested despite lack of evidence that polymerization was actually achieved.

Discussion

Receptors: Some general considerations.

Central to the clonal selection theory of the immune response is the process by which the immunologically competent, precommitted cell can recognize -- and thus be selected by -- specific antigenic determinants. Such a process must involve structures which interact with antigens with sufficient energy -- bind with sufficient affinity -- to initiate the cellular activities that are the immune response. These "recognition structures" are clearly crucial to the specificity and control of the immune system (Siskind & Benacerraf, 1969). They are referred to as "antigen-binding receptors".

In broad functional terms, a receptor is the mechanism by which a signal is perceived and a response initiated; it is the process which mediates the afferent interactions between a "black box" and its environment. In many biological systems, such as the immune response, this function may take the following structural form. The signal is a small molecule or a particular conformational determinant of a macromolecule. The signal-binding or recognition site is most likely to be a cleft or area of a macromolecule. The initial interaction, the "specific" binding of one molecule by another, may occur either outside the cell, at the surface membrane, or inside the cell. The interaction yields a bimolecular

complex of "higher informational content" which may directly stimulate the cell in a variety of ways, for example, by derepressing a gene, by activating an enzyme, or by serving as an enzyme substrate. Alternatively, these mechanisms may produce a second intracellular signal necessary to initiate the cell response. Thus, the receptor function may be structurally very complex.

Nevertheless, two components can be differentiated -- (1) the specific binding of the signal and (2) the mechanism by which that binding leads to cell activation. The processes relevant to the non-covalent binding of two molecules have been carefully studied, for example, in enzyme-substrate systems, and it is generally assumed that similar forces operate in signal-receptor systems. However, somewhat greater methodological difficulties are involved in elucidating the mechanisms and consequences of molecule-cell interaction. This is particularly true in systems such as the immune response, where the binding structure is believed to function at, and perhaps be incorporated into, the surface membrane of the cell. In no such systems has the mechanism of receptor function been clearly established.

This discussion will start with a consideration of humoral antibody, for which there is more knowledge about the relation between the structure and function of

the combining site. Next, the focus will be on the specificity of cell-mediated immunity, which is a functional property of cell binding of antigen, and then on structural and mechanistic aspects of the recognition site. In this context, affinity labelling and synthetic antigens will be considered as techniques for studying both functional and structural characteristics of the cell receptor in delayed hypersensitivity.

The Antibody Response.

Immunization with a classical hapten-protein conjugate elicits an antibody population which is tremendously heterogeneous both structurally and functionally (Haber, 1967; Rockey et al, 1970). A sub-population with specificity for the hapten can be demonstrated, and the average hapten affinity of this fraction increases progressively with time after immunization (Eisen & Siskind, 1964). This maturation phenomenon has been observed in two species with several different haptens (Haber et al, 1967). There is evidence both from immunological studies (Kabat, 1966; Haber et al, 1967) and from X-ray crystallography (Poljak et al, 1972), that the antibody combining site is approximately 25-44Å x 10-17Å x 6-10Å. This is a considerably larger volume than is occupied by a simple

hapten such as ϵ-DNP-lysine (Richards et al, 1967). Several studies indicate that determinants near the hapten are important in antibody binding, and that immune serum may also show maturation against the larger areas containing hapten and neighboring determinants (Parker et al, 1966; Henney, 1970). Thus, there can be an antibody population which is specific for regions containing both hapten and protein carrier determinants and which can bind either hapten alone or protein alone.

Strausbach et al (1972) have shown such antibodies to be physiologically significant. They demonstrated that in both in vivo and in vitro systems previously sensitized to hapten-carrier conjugates, carrier alone could elicit a "significant and strong" anti-hapten secondary response. The affinities of antibodies produced with stimulation either by hapten-carrier conjugates or by carrier alone, were comparable; however, the antibodies produced by carrier stimulation appeared to be slightly more heterogeneous in regard to hapten binding specificity.

However, several important classes of immunogens elicit antibody with no maturation of binding affinity and with markedly reduced structural heterogeneity. One class includes antigens composed of small, strong, immunodominant determinant located in the midst of a relatively featureless background of rather low

immunogenicity. For example, the defined sequence polymer of widely spaced ϵ-DNP-lysine residues on a D-L,alanine backbone (Richards et al, 1967; Richards et al, 1969) elicited antibody whose affinity for ϵ-DNP-lysine was initially high (K_A 10^9-10^{10}) and remained high, and which demonstrated restricted heterogeneity throughout. One interpretation is that the bland poly-alanine backbone apparently contributes minimally to the interaction with, and therefore selection of, antibody. Thus the antigen elicits only antibody which can bind very tightly to the sole available immunogenic determinant -- the ϵ-DNP-lysine "hapten".

Antibody responses of limited heterogeneity are also evoked by large, rigidly structured antigenic determinants of nearly sufficient size to fill an antibody combining site. Multiple examples of this have now been reported (approximate volumes of the antigen are given where possible): bradykinin (sphere, diameter 16Å) (Haber et al, 1967); vasopressin (19Å x 14Å x 11Å) (Wu & Rockey, 1969) ; folic acid (Rubenstein & Little, 1970); the "loop"-peptide of lysozyme (Maron et al, 1971); and $(DNP)_2$-gramicidin-S (Montgomery et al, 1972). The last two examples bear closer examination. The "loop"-peptide of lysozyme is a sequence of 24 amino acids, located on the outer surface of the intact enzyme. It contains one intrachain disulfide bond which presumably gives it

a relatively constrained configuration. Antibodies specific toward this region were prepared (1) by isolation from anti-lysozyme antisera with an immunoadsorbent containing loop-peptide or (2) by isolation on a lysozyme immunoadsorbent from anti-sera elicited by the loop-peptide conjugated to a non-immunogenic synthetic polypeptide. Antibodies to the loop region, prepared by these methods, showed restricted heterogeneity as compared to the totality of anti-lysozyme antibody population. Finally, unfolding of the peptide chain of the loop-peptide by various methods, resulted in a drastic decrease in the reactivity with specific antibodies. This study provides reassurance that the normal response to natural protein antigens includes sub-populations of antibodies directed against structured site-filling determinants, and that these sub-populations show restrictions of heterogeneity comparable to that found in the experimental situations cited.

$(DNP)_2$-gramicidin-S possesses a rigid, stable, cyclic structure with a 2-fold axis of symmetry, carrying two DNP groups in an identical conformational environment. It is complementary in size to the antibody combining site. Rabbits immunized with this antigen produced anti-DNP antibody of markedly restricted hetergeneity. One closely studied antiserum displayed a "monoclonal

isoelectric spectrum and a heterogeneity index of one" and was considered "biosynthetically homogeneous" (Montgomery et al, 1972). Thus a sufficiently structured antigen can apparently select and stimulate a single antibody-producing cell clone *in vivo*.

In summary, these studies support the following conceptualization of the humoral antibody immune response. First, although an antibody population may appear to be quite specific for a single small determinant (hapten), of a larger antigen (hapten-carrier conjugate), significant sub-populations may also have considerable interaction with adjacent determinants (i.e. on the carrier). These interactions may be crucial to the "classic" characteristics of an anti-hapten antibody response -- the heterogeneity of the antibody population and the maturation of its binding affinity. Larger, site-filling determinants elicit a restricted sub-population of antibodies with relatively high, uniform affinity. This effect is increasingly pronounced for determinants of greater conformational rigidity.

Functional aspects of the receptor: the specificity of cell-mediated immunity.

In contrast to the antibody response, the specificity of cell-mediated immunity is poorly characterized. This gap is largely a function of methodological

limitations. The product of B cells can be purified from serum by a variety of procedures and studied and manipulated with a wide range of techniques derived from protein chemistry. T cells generally must be obtained from lymphoid tissue or peritoneal exudates and require careful handling to survive. The purification of cell populations either by class (T *vs* B) or by antigen specificity, is still a major problem (Miller et al, 1971; Wigzell & Andersson, 1971). The state of sensitization of T cells, their specificity and their function, can only be studied through assay of the effects they can mediate consequent to activation by specific antigens. Until a few years ago, the only available assay was the skin test -- either on an immunized animal or on the recipient of sensitized cells (adoptive transfer). The relatively recent development of a host of *in vitro* assays has not, however, substantially improved certain aspects of the problem (Bloom & Glade, 1971; Revillard, 1971).

The assays themselves are difficult to perform and reproduce. Different assays are based on different effects mediated by antigen-activated lymphocytes, for example, inhibition of macrophage migration and cytolysis of target cells. Further, both *in vivo* and *in vitro* assays measure the total effect of a given cell population. It is possible that sub-populations of different

composition and size mediate different effects and that the responses observed are not proportional to the number of sensitized cells specifically stimulated by antigen. Thus, at present, the results of the various assays can be compared only semi-quantitatively. Finally the assays reflect cell activation which is the functional effect of receptor binding of antigen; they do not directly measure the relative affinity of the "recognition site" for various antigens, which is a structural property of the system. These issues must be considered when evaluating investigations of receptor specificity.

The earliest studies of the "immunological specificity of delayed hypersensitivity reactions to hapten-protein conjugates ... demonstrated the important contribution of the carrier protein ... " (Benacerraf & Levine, 1962; also Benacerraf & Gell, 1959a; Benacerraf & Gell, 1959b; Gell & Benacerraf, 1961). This was noted to contrast with the hapten specificity of the antibody response. The phenomenon of carrier specificity was soon examined using a remarkable diversity of carefully selected haptens, proteins, and conjugating procedures. The simplest <u>in vivo</u> system was used -- skin testing of sensitized guinea pigs. The following relationships were clearly established.

(1) "Sensitized animals which gave strong delayed reactions to the immunizing conjugate cross-reacted poorly or not at all to (a) conjugates of the same hapten with a different carrier protein or (b) conjugates differing from the immunizing conjugate by having an ε-amino-caproyl chain interposed between hapten and its attachment on the carrier protein. Animals sensitized with either lightly or heavily substituted conjugates exhibited strong delayed reactions to both conjugates, but more intense reactions to the immunizing conjugate were always observed" (Benacerraf & Levine, 1962).

(2) Animals sensitized to a hapten conjugated to guinea pig serum albumin showed varying degrees of cross-reactivity to the same hapten conjugated to other mammalian serum albumins. Such serum albumins are known to exhibit serological cross-reactivity. The same hapten on completely unrelated proteins failed to cross-react. The homologous conjugate always elicited the largest reaction, while "the order of the extent of cross-reaction of the other serum albumin conjugates differed from one animal to another, possibly owing to individual variation ... It is also noteworthy that the non-ionic hapten p-nitrobenzoyl shows strikingly more carrier-specificity than the ionic m-azobenzenesulfonate" (Gell & Silverstein, 1962a).

(3) "The specificity of the delayed hypersensitivity reaction is such that it can distinguish the type [and position] of linkage by which a given hapten is attached to a given protein carrier. In no instance was a cross-reaction observed in any of the hapten-protein pairs among which the only difference was the site of attachment of hapten on the carrier protein surface" (Gell & Silverstein, 1962a).

(4) An elaborate series of substituted azobenzene conjugates of guinea pig albumin were prepared, with the benzene ring containing various ionic and non-ionic groups in different positions. There was appreciable cross-reactivity between the hapten-protein pairs among which the only difference was the nature and/or position of the substituent on the

> benzene ring of the hapten. The degree of cross-reactivity was clearly greater than exhibited by anti-hapten antibody. As always, the immunizing hapten-carrier conjugate elicited the maximal response (Silverstein & Gell, 1962b).

Thus delayed hypersensitivity was shown to be not only "more specific with respect to the total function determinant, but less specific with respect to the small haptenic moiety" (Silverstein & Gell, 1962b).

With the development of *in vitro* assays, the specificity of cell-mediated immunity to hapten-protein conjugates has been carefully re-examined (Davie & Paul, 1970; Paul & Siskind, 1970; Paul, 1970; Rajewsky & Pohlit, 1971). The "classic" findings cited above have been emphatically confirmed and a somewhat more precise quantification of the degree of cross-reactivity among the various conjugates has been achieved. Further, Francis and Paul, (1970) have used an *in vitro* assay to study the ability of a hapten (ε-DNP-L-lysine) to inhibit the activation of sensitized cells by the immunizing conjugate (DNP-GPA). They concluded:

> "Inhibition can be demonstrated only when exceedingly small antigen concentrations (10^{-3}µg/ml) and enormous molar ratios of hapten (10^{4}) are used.
>
> These data suggest that cells involved in cellular immune responses possess receptors which can interact with haptenic determinants. The simple interaction of hapten with such receptors is, however, insufficient to stimulate such cells so that the specific stimulation of immunocompetent cells must involve other requirements".

Simple, reversible, structural interaction with the recognition site is clearly insufficient for functional activation of the receptor mechanism.

Small peptides of known amino acid composition and sequence, such as polypeptide hormones, have also been used to investigate the specificity of delayed hypersensitivity. For example, the immunogenic capacity of the ACTH 1-24 peptide appears to reside in the 11-24 fragment, much of it in the 17-24 octapeptide. Smaller peptides are clearly inactive. Comparison of antigenic fragments (eg. 11-24) with non-antigenic ones (e.g. 1-13) suggest that immunogenicity is a function of particular amino acid sequences rather than simply the presence of particular types of amino acids. The sequence of the peptide determines its conformation, which is, in turn, the basis of its immunologic activity (Savin & Liauw, 1967).

Another study using synthetic Angiotensin II (an octapeptide) demonstrated a close relationship between the immunogenicicity of a determinant and its capacity to elicit a delayed reaction. That is, (1) free angiotensin and angiotensin-protein conjugates were immunogenic and (2) in animals immunized with conjugates, both free angiotensin and heterologous conjugates could cause delayed reactions (Dietrich, 1966). Unfortunately there is no detailed data on the relation between

peptide sequence and conformation in these systems.

The specificity of cell-mediated immunity to the DNP-oligo-lysine system has been exhaustively examined. The original *in vivo* studies showed that small non-immunogenic members of the homologous series of peptides elicited only antibody-mediated (Arthus) reactions. Larger, immunogenic members elicited both Arthus and delayed reactions. In particular, the upper limit of the antibody combining site corresponded to the smallest peptide which was immunogenic (Schlossman & Levine, 1967). More recent *in vivo* work has demonstrated "the high degree of specificity possessed by the recognition mechanism associated with the lymphoid cells which mediate cellular immune responses" (Paul et al, 1971). In particular,

> "the sensitized cell is maximally stimulated by the homologous immunizing antigen and can discriminate among components which differ from one another only in the position of a dinitrophenyl group or D-lysine residue on an identical oligo-lysine backbone ... [it appears that] the majority of cells produced as a consequence of immunization have stereo-specific antigen receptors for the DNP-oligo-lysine [i.e. the hapten-carrier conjugate] used to induce the response; a smaller and more variably-sized population of cells is produced with receptors specific for the oligo-lysine portion [i.e. carrier] of the immunizing antigen" (Schlossman et al, 1969).

One of these studies found an imperfect correlation between the *in vivo* skin test and the *in vitro* assay. In particular, the assays indicated reactivity to some

antigens which had given negative skin tests. The pattern of these responses suggested the existence of a smaller, purely carrier-specific cell population, as mentioned above. The investigators commented only that "these observations suggest that the in vitro response as measured by thymidine incorporation is either a more sensitive assay, or alternatively, not a simple in vitro correlate of delayed hypersensitivity" (Schlossman et al, 1969).

The studies considered thus far have focused on the specificity of delayed hypersensitivity at a given point in the immune response. There remains the important issue of whether, with time, delayed hypersensitivity exhibits a maturation of affinity and broadening of specificity analogous to the changes seen in the antibody response. The direct in vivo system is not well suited to approaching this question. The onset of antibody production causes the appearance of "immediate hypersensitivity" (the Arthus reaction) at the site of an intradermal challenge. As expected, this reaction is hapten-specific and it generally extends from about 1 to 6 hours after the challenge. Its appearance complicates, at the very least, the reading and interpretation of the delayed reaction. In most in vivo studies, skin testing was performed 7 - 10 days after

immunization, at which time delayed hypersensitivity can be demonstrated but there is generally no Arthus reaction. This procedure avoids the problem but, unfortunately, begs the question.

One investigation of this issue used the adoptive transfer system (Phair & Kantor, 1968). This work indicated that "the ability of donor cells to transfer delayed hypersensitivity persisted throughout the primary and secondary antibody responses of the donor animals. The skin reactions elicited in recipients of cells from hyperimmunized guinea pigs remained conjugate-specific throughout the immune response".

Several studies have attempted to evaluate directly the course of the delayed hypersensitivity response in an actively sensitized animal (Henney, 1970; Kruger et al, 1971). These studies appear to indicate that after 2 - 3 weeks a hapten-specific component of the delayed reaction can be identified. This component was accented by challenge with higher doses of hapten on a heterologous carrier. In all cases the simultaneous presence of hapten-specific antibody was documented either by the Arthus reactions or serum binding activity.

However, the conclusions reached in these studies have been challenged. Using passively and actively immunized guinea pigs, Phair and Kantor (1970) studied

the interaction of sensitized cells, immune serum and challenge antigen at various concentrations. Hapten-specific delayed reactions were produced in the recipients of sensitized cells by (1) challenge with a high concentration of hapten conjugated to heterologous carrier protein; (2) simultaneous transfer of hyperimmune serum; (3) challenge at the site of an unrelated immediate reaction. In actively immunized guinea pigs, hapten-specific reactions were facilitated by preparation of the challenge site with anti-hapten antibody, normal serum, or unrelated immediate reactions. The investigators considered that two mechanisms might underlie such hapten-specific delayed reactions:

> " (1) increased retention of antigen at the skin site secondary to antigen-antibody interaction and consequent stimulation of those cells capable of responding to an increased concentration of hapten; (2) a non-specific increase in the number of mononuclear cells at the reaction site, thereby increasing the probability of interaction of antigen and cells with a high affinity for the haptenic group" (Phair & Kantor, 1970).

The question of "maturation" of the specificity of delayed hypersensitivity has also been examined using an <u>in vitro</u> assay (Henney & Nordin, 1971). The results obtained will be considered in some detail, for they highlight both the potential and the problems of <u>in vitro</u> methodologies (Table II). The data for effector

Table II

Specificity of cytotoxic lymphocyte population from guinea pig spleen following immunization with DNP-HGG

Time after Immunization	%Specific ^{51}Cr Release[a] from Mastocytoma cells Coupled with:		
	DNP-HGG	DNP-BSA	HGG
days			
4	4.1	0	0
7	13.5	4.5	7.1
14	31.3	20.0	28.6
21	19.9	15.9	17.6
28	18.7	10.1	15.9

[a] %^{51}Cr release in the presence of lymphocytes from specifically immunized animals minus the release obtained in the presence of lymphocytes from unimmunized controls.

cell activation by the immunizing conjugate confirm previous expectations. The authors comment that it was "rather unexpected" to find

> "that 'target' cells bearing only the carrier portion of the immunizing conjugate were lysed as effectively as those bearing the whole conjugate".

They used the macrophage migration inhibition test to provide additional evidence. Those results reassured them (Table III). Nevertheless, it is quite clear that the two assays give quantitatively different anwers. Moreover, although the workers make no comment on it, the degree of "hapten-specific" lysis (64% of conjugate lysis on d.14; 80% on d.21) is not only unexpected, but quite startling. The cause of these anomalies is uncertain. First, the stimulus is very complex. Random light conjugation of a population of carriers could easily produce overlapping sub-populations containing (1) each major carrier determinant intact and (2) the hapten in a wide variety of environments. Further, the in vitro assays probably are more sensitive than the in vivo test and can more readily detect small populations of cells responsive to pure carrier determinants or hapten-focused cross-reacting determinants. There are also probably "non-specific recruitment" effects. In addition, a variety of cell types are present in the lymphocyte pool assayed. For instance, the authors

Table III

A comparison between the specificity of the humoral and cell-mediated immune response of guinea pigs 14 days following immunization with dinitrophenylated human IgG

	DNP-HGG	DNP-BSA	HGG
% cytolysis[a]	31.3	20.0	28.6
% inhibition of macrophage migration[b]	60 ± 2.2	24 ± 4.8	41 ± 2.8
PFC/10^6 splenic lymphocytes[c]	930	789	6

[a] %specific ^{51}Cr release as per Table II

[b] The percent inhibition of the migration of peritoneal exudate cells from unimmunized guinea pigs in the presence of lymphocytes, from animals immunized with DNP-HGG, and 300g/ml antigen. The values given are ± standard error of the mean of nine determinants on a pooled lymphocyte preparation from three spleens.

[c] Total ("direct" and "indirect") plaques per 10^6 splenic lymphocytes using antigen-coated SRC.

clearly document cells producing hapten- and carrier-specific antibody, but do not control for any effect this might have. It must be stressed that this study is one of the most careful and precise available; it is the state of the art that wants improving.

One well-investigated system does seem to exhibit hapten-sepcific delayed hypersensitivity -- p-azobenzene-arsonate (pABA) derivatives of tyrosine (Leskowitz, 1963; Leskowitz et al, 1966). Studies with these derivatives emphasise the relationship between the immunogenicity of a given molecular configuration and its capacity to evoke a delayed reaction. Animals immunized with pABA-N-acetyl-L-tyrosine amide (pABA-tyr; molecular weight, 450), the smallest known independent immunogen, will produce characteristic *in vivo* delayed reactions to homologous or heterologous conjugates of pABA (Leskowitz et al, 1966; Borek, Stupp & Sela, 1965). *In vitro*, their lymphocytes can be activated by pABA-tyr or a protein conjugate with equal efficiency (Nauciel & Raynaud, 1971). Further, the immunogenicity of a variety of non- or weakly-immunogenic proteins and synthetic polypeptides is notably increased by conjugation with pABA. This change is accompanied by the development of hapten-specific delayed sensitivity (Jones & Leskowitz, 1965; Borek & Stupp, 1965; Stupp, Borek & Sela, 1966).

Of the azophenyl acids, only conjugates of phosphanilic acid (pABP) share this immunologic property; conjugates of aminobenzoic (pAB) or sulfanilic (pABS) acids clearly do not. (Leskowitz & Zak, 1966; Borek & Stupp, 1965). Substituted derivatives of pABA and pABP conjugates have been used to compare the cross-reactivity of hapten-specific delayed sensitivity with that of hapten-specific antibody. The results were interpreted as indicating that, in this system at least, "recognition sites for delayed sensitivity are very similar to those for antibody" (Leskowitz, 1967). Nevertheless, the mechanism of hapten-specific delayed hypersensitivity remains unclear. The simplest hypothesis is that this particular configuration of polar and hydrophobic groups can by itself provide sufficient interaction to activate the necessary and appropriate cells. That is, unlike other haptens, this moiety does not require the additional energy contribution of adjacent carrier determinants.

Structural Aspects of the Receptor.

The work reviewed to this point has concerned the specificity of delayed hypersensitivity and, therefore, the functional characteristics of the antigen receptors involved. There has also been a considerable effort to clarify the structural characteristics of T cell

receptors. Here too, there are a variety of methodological problems. The lymphocyte populations most easily obtained contain more B cells than T cells. Moreover, B cells appear to bind much more antigen to their surface, and more readily. Therefore, techniques are required which specifically detect T cells or provide T cell enriched populations (Miller et al, 1971). Much of the work available is very recent and either unconfirmed or challenged. This survey will focus on those generalizations that seem most broadly supported.

Multiple lines of evidence suggest that T cells do have specific antigen binding receptors: (1) the specific activation of T cells by antigen; (2) specific inactivation of T cells by ^{125}I-labelled antigen (antigen induced cell "suicide"); (3) the capacity of T cells to carry memory and to be made tolerant (reviewed by Möller, 1970; Dwyer & Mackay, 1971; Miller et al, 1971). It has been somewhat difficult to establish conclusively that there are antigen-binding structures actually on the cell surface. T cells seem to have far fewer such structures than B cells (Raff, 1970; Rabellino et al, 1971; Unanue et al, 1971; Greaves & Hogg, 1971). The estimate is that there are in the order of 500 receptors on the surface of a T cell, but 50,000 - 150,000 on a B cell (Marchalonis et al, 1972).

Evidence is accumulating that the recognition structure is an immunoglobulin: (1) Anti-light-chain antisera has been reported to inhibit the ability of lymphocytes to effect the graft vs host reaction of the transfer of delayed hypersensitivity (Greaves et al, 1969; Mason & Warner, 1970); (2) anti-kappa antisera has been shown to protect T cells against ^{125}I-antigen "suicide" (Basten et al, 1971); (3) anti-Fab and anti-light-chain antisera can inhibit T-rosette forming cells (Greaves & Hogg, 1971).

The class of heavy chian is a matter of considerable debate. Many studies have found no evidence for any of the currently known class of heavy chain (reviewed by Greaves & Hogg, 1971). Recent data from Greaves and Hogg (1971) suggest that there are μ chains on T cells but perhaps only partially exposed. They support the interpretation that the differences between antigen receptors on T and B cells are both (1) quantitative, T cells having far fewer receptors, as noted above, and (2) qualitative, T cell receptors being "buried in the plasma membrane in such a way that only the variable region (containing the combining site) is exposed". Furthermore, speculating from studies of cells before and after challenge, they constructed a model of maturational changes in the relationship between the receptor

and the cell surface membrane. In particular, they hypothesized increased exposure of the heavy (μ) chains after immunization. Other evidence for μ chains in the receptor is based on the claim that immunoglobulin isolated from the surface of T cells contains heavy chain which resembled μ-chain in electrophoretic behavior (Marchalonis et al, 1972). Both these studies are unconfirmed and involve new, specialized techniques; they are, therefore, difficult to analyze critically at this time.

It must be stressed that the evidence associating the immunoglobulin detected on the cell surface with the putative receptor is subject to several important limitations. First, there is no unequivocal evidence that the cell has synthesized the immunoglobulin. However, there have been several attempts to coat lymphoid cells with "exogenous" antibody using a variety of in vivo and in vitro conditions and none have produced any significant passive absorption (Greaves, 1971). Second, the inhibition of receptor function by anti-light-chain or anti-Fab antisera may result from (a) the receptor site actually containing the immunoglobulin determinant or (b) the receptor site being in closer proximity to the determinant and the binding of antisera interfering with the receptor by steric hindrance. Moreover, the

techniques currently stressed cannot clarify antigen-binding specificities and affinities of the recognition site nor elucidate the mechanism by which antigen-binding activates the cell.

Some Hypotheses about the Receptor.

Extrapolating from the wide variety of data that has been presented, it is possible to formulate several hypotheses about the antigen-binding structure of cell-mediated immunity. This may facilitate considering methods for further study. (For convenience, "receptor" will be used when referring to the antigen-binding structure, which is the first component of the functional receptor).

(1) The receptor is an immunoglobulin.

This will only be established when the receptor is actually isolated. The class of heavy chain is of little concern for this model. It would not, however, be surprising if a new class were found. The Fc region of a cell-associated receptor could reasonably have a different structure for its special function.

(2) The receptor site is the same size as an immunoglobulin combining site.

(3) An "effective determinant" is one which can specifically elicit a cell-mediated immune reaction. It is generally

about the size of the receptor site.

As has been seen, the volume of the combining site is considerably larger than most simple haptens and can encompass significant portions of most of the globular proteins commonly used as carriers. Data from several systems shows that (a) the minimum size for an immunogen is approximately the upper limit of the antibody combining site and (b) the requirements (including size) for immunogenicity are closely related to those for eliciting a delayed reaction. Further, there is evidence that an antibody combining site may have several "contact regions" each with a different "specificity" (Rosenstein et al, 1972). Presumably, therefore, a site-filling antigen would bind to the site at more than one point. As a result of such multiple contacts, it might be expected that (a) the affinity of the site for the antigen would be relatively great (in the antibody systems considered above K_o's of about 10^6 to $10^9 (M/L)^{-1}$ are generally found), (b) altering any single determinant (eg.hapten) would not greatly affect the overall interaction of the antigen and the site unless marked steric hindrance was introduced and (c) the relative configuration of the various contact determinants (i.e. the structure of the antigen) would be of central importance to the overall interaction. These characteristics anticipated of a site-filling determinant are congruent with the phenomena of

carrier specificity.

(4) As carrier specificity is both sufficient and necessary in cell-mediated immunity, such site-filling is a requirement for activation of the cell. One can only speculate about the mechanism of such a receptor. It might reasonably be offered that the interaction of antigen and receptor causes a conformational change in the receptor which leads to cell activation. Metzger (1970) has reviewed the work that might provide data to support such a suggestion. He concluded:

> "So far, no convincing evidence is at hand which demonstrates that reactions at the combining sites of antibody produce defined conformational changes elsewhere in the molecule".

It could be argued that the environment of the cell receptor facilitated changes not noticeable in other contexts. Moreover, the systems studied have primarily involved either highly heterogeneous antibody populations against a hapten-protein conjugate, or myeloma proteins with anti-hapten binding activity (Ashman et al, 1971). The next logical system to examine would seem to be the structured site-filling antigens which elicit antibody populations of restricted heterogeneity.

The model suggests an explanation for why some "structure" -- a "preferred conformation" -- seems to be a requirement for immunogenicity. The process of inducing a conformational change in the receptor requires

energy. One source for this energy might be the potential energy inherent in a preferred structural configuration of the antigenic determinant. However, with increasing rigidity, the antigen could presumably fit into fewer receptor sites. This may explain such situations as the $(DNP)_2$-gramicidin-S system where only one clone was stimulated in some animals, and apparently none in others.

Exceptions to the site-filling structured antigen hypothesis can be accommodated by the model by presuming that they can satisfy in other ways the requirements for energy of interaction with the receptor. For example, the "hapten-specific" delayed hypersensitivity of the pABA system may represent a situation where an ordered arrangement of strong polar and hydrophobic groups can interact sufficiently with particular points around the receptor combining site to cause activation. Finally, it should be noted that this model is not intended to apply equally to the interactions of antigens and antibody-producing cells. While there is evidence that these hypotheses might be relevant to some aspects of that system, different mechanisms may operate in other aspects.

Methods for the Study of the Receptor.

More direct and controlled methods are required to characterize further the structure and function of the

receptor and, ultimately, to isolate it from the cell. "Affinity labelling" is a technique which has been very successfully used in the study of the antibody combining site (Singer & Doolittle, 1966; Singer, 1967). The method depends upon the ability of a bifunctional reagent to (1) combine specifically with the particular antibody and (2) react readily with amino acid residues to form covalent bonds. The successful labelling of the receptor recognition site should block the ability of the cell to respond to antigen. This functional effect could be evaluated with an in vitro assay or an adoptive transfer. Necessary controls would include (1) persistence of sensitivity to an unrelated antigen to evaluate non-specific toxic effects of the procedure and (2) "cross-over" experiments using labels derived from two unrelated determinants to evaluate effects due to possible differences in the numbers and types of sensitized cells elicited by different antigens. Further, a radioactive tag on the affinity label would permit the use of autoradiography to localize the site of the receptor, and would provide a means of tracing the labelled recognition structure through physico-chemical procedures leading to its isolation and structural characterization. In these experiments it would be necessary to evaluate the specificity with which the label attached to the receptor as opposed to elsewhere on the cell. This might be done

by protection and differential labelling techniques (Singer, 1967).

Affinity labelling of receptors on antibody-producing cells has been attempted and is fraught with problems. Plotz (1969) and Segal et al (1969) independently reported the specific inhibition of antibody response using affinity labels derived from hapten. This work is often cited, but not confirmed and apparently not reproducible. Naor et al (1970) demonstrated that such systems are plagued by many effects not related to the reaction of the label with the receptor. Moreover, they concluded that the percentage of specifically sensitized cells in a normal lymphocyte population was so small and the fraction of specific receptor to cell membrane so tiny that "the cumulative level of nonspecific modifications in the system almost certainly would nullify specific approaches to isolating few affinity labelled receptors".

In cellular immune systems, the difficulties of affinity labelling are compounded. Kantor (1969) was unsuccessful in attempts to use Segal's affinity labels to block the transfer of delayed hypersensitivity even though the treated cells were heavily conjugated. In contrast, Hill (1971) claimed that an affinity label derived from the same hapten could block an adoptive transfer and could permit the isolation of a putative receptor.

The immunizing agent Hill used was 1-Fluoro-2,4-dinitrobenzene (FDNB) (I in Fig. 1), which is known to react with the free amino groups of lysines of skin proteins to yield ϵ-2,4-dinitrophenyl lysine (ϵ-DNP-lysine) residues (II). It is probably by thus altering autologous proteins that FDNB causes contact sensitivity. Sensitization with FDNB or DNP-protein conjugates also effectively elicits anti-DNP antibodies. The binding sites of these antibodies have been extensively investigated with a series of affinity labels, the most successful being m-nitrobenzenediazonium fluoroborate (MNBDF) (III) (Good, Traylor & Singer, 1967).

Hill sought to derive from ϵ-DNP-lysine a new affinity label which would "resemble the DNP-protein conjugate more closely". He reports the synthesis of ϵ-2-nitro-4-aminophenyl lysine (ϵ-NAP-lysine) (IV) and its conversion to ϵ-2-nitro-4-diazophenyl lysine (ϵ-NDP-lysine). He further claims that the precipitation of anti-DNP antibody by DNP-albumin was blocked reversibly by ϵ-NAP-lysine and irreversibly by the affinity label. No data is presented to support these assertions. Previous investigation of hapten-antibody binding in the DNP system seemed to indicate that reduction of a nitro group, particularly the "lead" or "4" group, would significantly lower the affinity of the combining site for the altered hapten (Pressman & Grossberg, 1968).

Our attempts to reproduce Hill's work strongly indicated that this was the case and, threfore, affinity labelling of cells with this system was not attempted.

The work of Francis and Paul (1971) and of Kantor (1969) suggests that most hapten-derived affinity labels will be ineffective because of insufficient ability to localize at the receptor. Rather, an affinity label for the recognition site of delayed hypersensitivity should be based on an "effective determinant". In particular, it would be desirable to have a small, defined, readily synthesized determinant to which the reactive labelling group could be easily attached; possibilities include immunogenic DNP-oligo-lysines, hormone peptide fragments, or pABA derivatives. Another model might be the defined sequence antigen of widely spaced ϵ-DNP-lysines on an alanine backbone (Richards et al, 1967). This polypeptide readily elicited the production of anti-DNP antibody in rabbits, but would not elicit delayed hypersensitivity in guinea pigs (Leskowitz, 1967). There are many advantages to using a peptide polymer of this type as an immunogen. Those discussed by Richards et al include simplicity, homogeneity and conformation. With respect to the current issue, the monomer or a similar sub-unit of the peptide might serve as the basis for an affinity label. In addition, controlled variations could be readily made for use in studying the specificity of the receptor (as per Parker et al, 1966).

The task thus became to establish the minimal modification necessary to make the above peptide immunogenic to guinea pigs. The requirements for immunogenicity of synthetic peptide antigens in terms of complexity of amino acid composition and sequence have been partially but not systematically studied (Sela, 1966; Borek, 1968; Rüde, 1970). On the basis of available reports it was felt that the addition of approximately two tyrosine residues among the ten alanine residues might be sufficient. There were also advantages to adding tyrosine -- (1) it could serve as the point of attachment of other haptenic groups, in particular, the azophenyl acids; (2) it could be radioactively tagged at very high specific activity with ^{125}I for a variety of studies. Unfortunately the synthesis of the desired polymer was not achieved due to time limitations and, therefore, no definite conclusions about its immunogenicity can be made. As detailed above, the monomeric units which were synthesized were non-immunogenic.

Summary.

Cell-mediated immunity encompasses many immunological phenomena including homograft and tumor rejection. The operative immunocompetent cells appear to be stimulated by the specific interaction of

antigen with surface membrane associated receptors. Evidence concerning the functional and structural characteristics of these receptors has been reviewed. Further study of them through an affinity label based on a defined peptide antigen has been considered. The molecular mechanism of the specific activation of immune cells is at present unclear, and a variety of methodological approaches will be necessary to further our understanding of antigen receptors.

Appendix

Details of Peptide Synthesis.

Four different procedures were used in the attempt to synthesize the desired peptide. The conditions which were varied included the initiator, the solvent for the initiator, the amounts of NCAs and the reaction time. The products were coded as follows: SAM-4A means Synthetic Antigen Model - procedure #4 - peak A.

Table AI compares the conditions of the various procedures. Figures A1, A2 and A3 show the gel chromatographic separation of the products of reaction and give the average peptide composition for the analyzed peaks.

Several observations may be made relating the various reaction conditions with the products obtained. First, initiation with ϵ-DNP-L-lysine HCl gave a very heterogeneous collection of products even when it was relatively well dissolved. We assumed that this reflected the tendency of the initiator amine to be protonated. Since only the free base can initiate NCA polymerization, different subsets of the initiator pool activated the NCAs at different times. This led to the formation of populations of different size peptides as reflected in the multiple peaks. Thus, it appeared necessary to prepare an initiator with an unproponated amino group. It should be noted that although SAM-2E had the desired composition,

its yield was so small and its homogeneity seemed so improbable that the procedure was not acceptable.

Initiation with the free base ethyl ester was much more satisfactory. Procedure 3 contained anomolous peaks of high tyrosine:DNP content because of technical errors in preparing the NCA reaction mixture. These led to the premature decomposition of some NCA-tyrosine and thus the presence of peptides initiated by tyrosine instead of DNP-lysine. Procedure 4 was almost ideal. Virtually all the product was found in a single, large, apparently homogeneous peak whose composition closely reflected that of the starting materials. The few small trailing peaks presumably reflect delayed initiation by the initially insoluble portion of initiator. With some additional experimentation, conditions might be found which give a reaction product of uniform composition. By thus eliminating the multiple purification steps, sufficiently high yields might be obtained to achieve successful polymerization of the peptide.

TABLE A1

Conditions of Peptide Synthesis Procedures

Procedures	NCA-D,L-ala	NCA-L-tyr	Initiator (Solubility)	Initiator Solvent	Reaction Time	Initial Composition (NCA-DL-ala : NCA-L-Tyr : Initiator)
1	300mg; 2.6mmol	120mg, 0.58 mmol	ε-DNP-L-lys HCl 101mg, 0.29 mmol (poor)	dioxane 3ml	2.0hr	9 : 2 : 1
2	460mg, 4.0 mmol	207mg, 0.75mmol	ε-DNP-L-lys HCl 87.5mg, 0.75mmol (good)	THF 3ml	4.0 hr	16 : 5 : 1
3	575mg, 5.0mmol	228 mg, 1.10mmol	ε-DNP-L-lys ethyl ester 113mg, 0.33 mmol (poor)	dioxane 4ml	12.0 hr	15 : 3.3 : 1
4	484mg, 4.2mmol	184 mg, 0.90mmol	ε-DNP-L-lys ethylester 105mg, 0.30 mmol (good - see text)	benzene - dioxane 3.0ml - 0.5ml	12.0hr	14 : 3 : 1

Figure A1

SAM-1 : Gel Chromatography Fractionation

OD_{360} vs Tube number

Conditions: P-2 Bio Gel column (2.5cm x 77cm) eluted with PBS. Each tube represents 9.9min collection (approximately 4.6ml).

Results : By amino acid analyzer

Peak (Tubes)	Average Composition (D,L-ala:L-tyr:ε-DNP-L-lys)
SAM-1A (61-70)	2.7 : 1 : 1

Figure A1

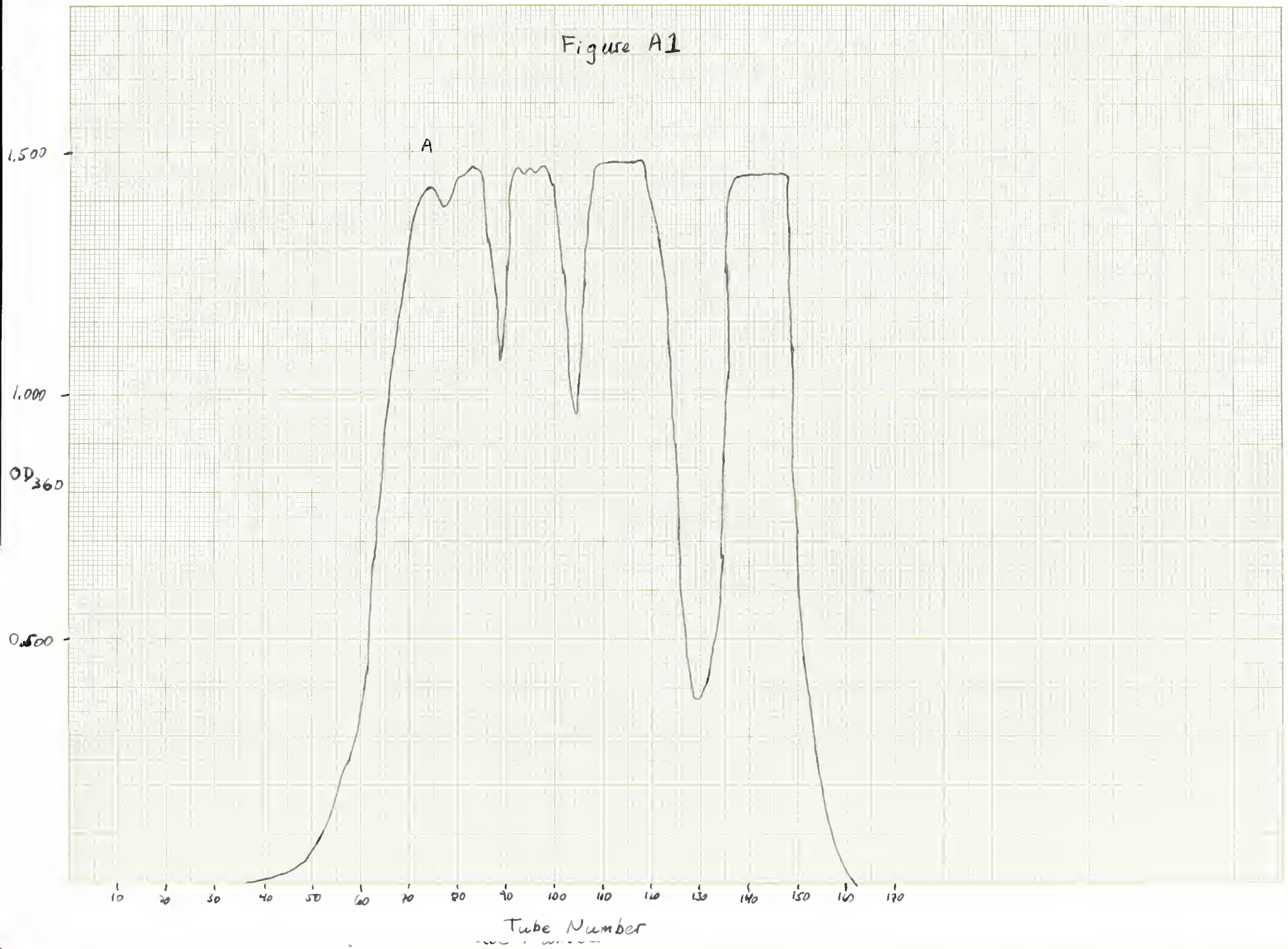

A
1.500
1.000
OD360
0.500
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
Tube Number

Figure A2

SAM-2 : Gel Chromatography Fractionation

OD_{360} vs Tube number

Conditions : as for SAM-1

Results : By amino acid analyzer

Peak (Tubes)	Average Composition (D,L-ala:L-tyr:ε-DNP-L-lys)
SAM-2A (29-31)	43 : 4.8 : 1
SAM-2E (53-60)	10.8 : 2.3 : 1

Figure-A2

1.500
1.000
OD_{360}
0.500
A
B
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
Tube Number

Figure A3

SAM-3 : Gel Chromatography Fractionation

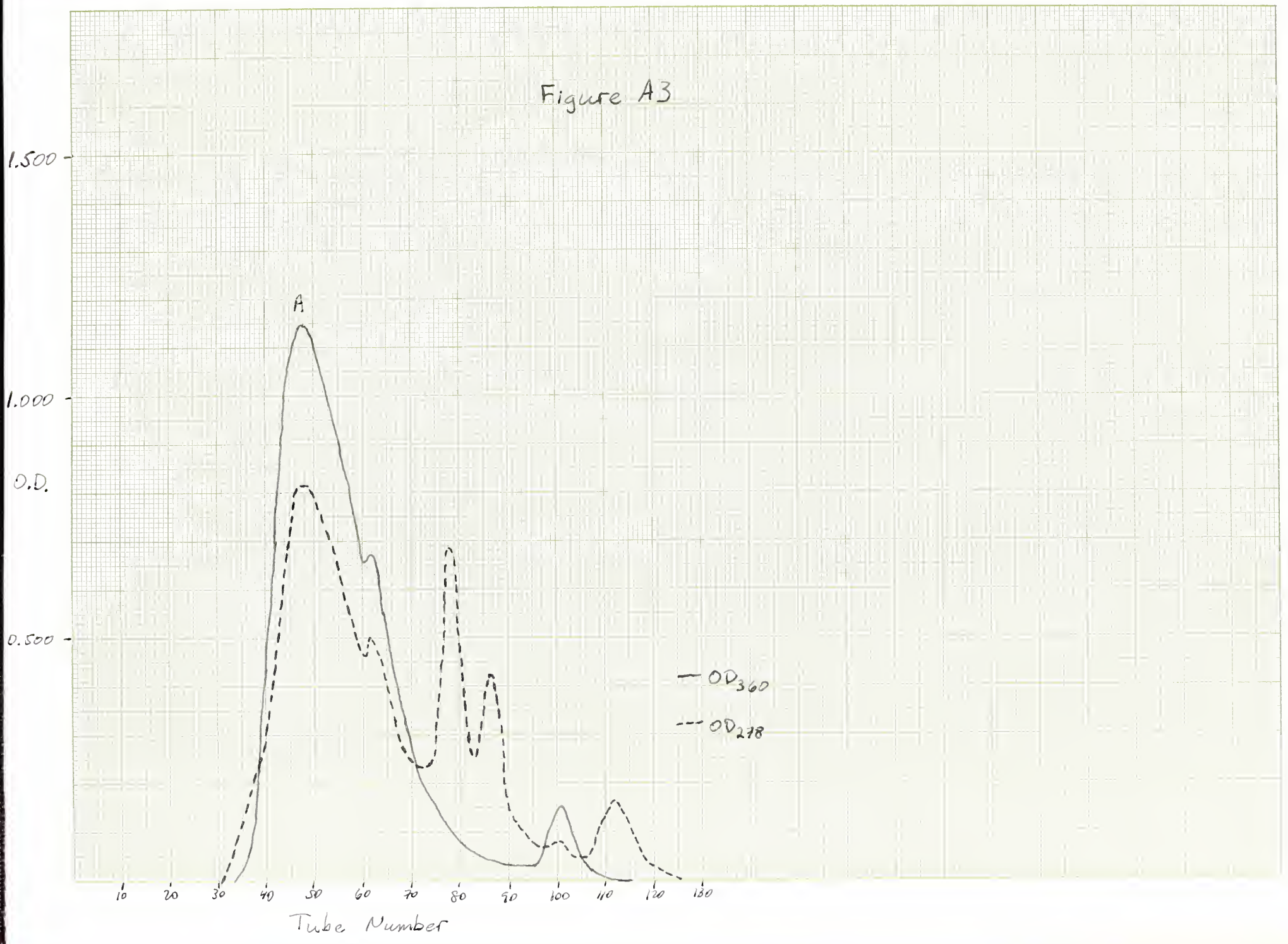
OD_{360} and OD_{278} vs Tube number

Conditions : as for SAM-4

Results : By spectrophotometric analysis

Peak (Tubes)	Average Composition (D,L-ala:L-tyr:ε-DNP-L-lys)
SAM-3A (41-66)	17.4 : 4 : 1

Figure A3


1.500
1.000
0.500
O.D.
A
10
20
30
40
50
60
70
80
90
100
110
120
130
Tube Number
OD360
OD278

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